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1   **Next-generation sequencing characterization of the gut**  
2   **bacterial community of gilthead sea bream (*Sparus aurata*,**  
3   **L.) fed low fishmeal based diets with increasing soybean**  
4   **meal levels**

5

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25   community, Next-generation sequencing, Growth, Gut

26 histology.

27

28   **Abstract**

29   The present study was carried out to evaluate growth, gut  
30   histology and gut bacterial community of gilthead sea bream  
31   (*Sparus aurata*) fed with increasing dietary soybean meal  
32   (SBM) levels in a low fishmeal (FM) based diet, in comparison  
33   with a control diet. Five isoproteic and isolipidic experimental  
34   diets were formulated to contain increasing levels of SBM (0,  
35   100, 200, and 300 g kg<sup>-1</sup> named S0, S10, S20 and S30,  
36   respectively) with 150 g kg<sup>-1</sup> of FM, and one control diet (C)  
37   without SBM and containing 350 g kg<sup>-1</sup> of FM. Sixty sea bream  
38   (initial body weight 75.9 ± 1.9 g, n = 900) per tank were reared  
39   in a recirculation system at 23.0 ± 1.0 °C and fed to satiation.  
40   The trial was run in triplicate and lasted 100 days. At the end of  
41   the trial fish fed the S30 diet showed a higher ( $P \leq 0.05$ )  
42   specific growth rate (SGR) compared to S0 (SGR, 1.17 ± 0.03,  
43   1.20 ± 0.01, 1.22 ± 0.01, 1.25 ± 0.01 and 1.21 ± 0.04 for S0,  
44   S10, S20, S30 and C, respectively), and a higher feed intake  
45   (FI) compared to S0, S10 and S20. Sea bream fed the C diet  
46   had a higher ( $P \leq 0.05$ ) FI compared to S0 (FI, 1.40 ± 0.01,  
47   1.45 ± 0.01, 1.44 ± 0.03, 1.51 ± 0.03 and 1.46 ± 0.02 for S0,  
48   S10, S20, S30 and C, respectively). No significant differences  
49   in feed conversion rate, protein efficiency ratio, gross protein  
50   efficiency and gross lipid efficiency among the treatments were  
51   detected. No specific histopathological changes indicative of  
52   soy-induced enteritis were observed in the intestine of any fish

53 examined. Gut bacterial community of the distal intestine  
54 content was analyzed by Next-Generation Sequencing. At the  
55 phylum level, the gut bacterial community was dominated by  
56 *Firmicutes* (relative abundance 71%), while the most  
57 represented family was *Lactobacillaceae* (26%). Even if no  
58 significant differences ( $P \leq 0.05$ ) in the gut bacterial  
59 community  $\alpha$  and  $\beta$ -diversity according to the different diets  
60 were detected, *Cyanobacteria* and *Lactobacillaceae*  
61 progressively increased from diet C to diet S30. In conclusion  
62 results of growth, nutrient utilization, gut histology and gut  
63 bacterial community indicate that SBM can be successfully  
64 incorporated up to a level of 300 g kg<sup>-1</sup> with the inclusion of  
65 150 g kg<sup>-1</sup> of FM, without any deleterious effects on growth,  
66 protein utilization and gut health during the on-growing of sea  
67 bream.

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## 77    **1. Introduction**

78

79        Gilthead sea bream is one of the most important species for  
80    European aquaculture, representing around 51% of the total  
81    finfish marine production in the Mediterranean area (FAO,  
82    2010). Due to the current economic downturn and the  
83    fluctuation of the gilthead sea bream market, a reduction in  
84    feed costs while ensuring optimal growth and fish health is  
85    essential to maintain the profitability of its farming (Martinez-  
86    Llorens et al., 2009; Mongile et al., 2014). In this context, the  
87    importance of vegetable protein is well recognized by feed  
88    industry operators due to the growing pressure for alternative  
89    fishmeal (FM) substitutes in fish diets. Among the different  
90    ingredients, soybean meal (SBM) is one of the most interesting  
91    alternative FM because of the advantages of supply, price, and  
92    protein and amino acid composition (Bonaldo et al., 2008).  
93    However this ingredient may induce a variety of histological  
94    and functional changes in the gastrointestinal tracts of fish,  
95    especially in salmonids, including morphological alterations  
96    and inflammation (Krogdahl et al., 2003, 2010). These changes  
97    may be due to direct effects of anti-nutritional factors in plant  
98    ingredients and/or the indirect result of diet-induced changes in  
99    the structure and function of the intestinal bacterial community  
100    (Olsen et al., 2001; Ringø et al., 2006).

101 Previous studies on gilthead sea bream have shown that the  
102 optimum dietary SBM levels, using a dietary FM content  
103 higher than 200 g kg<sup>-1</sup>, were 205 g kg<sup>-1</sup> for maximum growth  
104 (Martinez-Llorens et al., 2009). Further increasing the level of  
105 SBM up to 300 g kg<sup>-1</sup> of the diet had no significant effects on  
106 the specific growth rate (SGR), feed intake (FI) and feed  
107 conversion rate (FCR) in juvenile specimens of the same  
108 species, although high SBM level led to some changes in the  
109 distal intestine, with the presence of cellular infiltration of the  
110 submucosa and lamina propria (Bonaldo et al., 2008).

111 In this context the exploration of fish gut bacterial  
112 community can represent an emerging tool to evaluate the  
113 application of vegetal ingredients in fish feed formulations.  
114 Increased knowledge of the human gut microbiota is driving  
115 research into development, immunity, disease, lifestyle and  
116 nutrition (Furusawa et al., 2013). Similarly, the knowledge and  
117 manipulation of the gut microbiome in teleosts, especially in  
118 aquaculture, could be potentially addressed through nutrient  
119 digestion, synthesis, absorption, pathogen resistance, growth,  
120 sexual maturation, morphogenesis and survivorship (Llewellyn  
121 et al., 2014). To date, our understanding of the teleost gut  
122 bacterial community and of its functional significance has  
123 lagged well behind that of humans and other terrestrial  
124 vertebrates (Ray et al., 2012). Most understanding of the  
125 intestinal microbiota of fish is largely derived from culture-

126 based approaches and 16S rRNA gene fingerprinting methods  
127 such as denaturing gradient gel electrophoresis (DGGE).  
128 However, these methods usually reveal only a limited range of  
129 microbial diversity (Desai et al., 2012; Carda-Diéguez et al.,  
130 2014). Next-Generation Sequencing (NGS) has been used in  
131 recent years to examine the gut microbiome of humans,  
132 terrestrial and marine vertebrate including some fish species as  
133 recently reviewed by Ghanbari et al. (2015). However, only for  
134 a few species such as rainbow trout *Oncorhynchus mykiss*,  
135 Siberian sturgeon *Acipenser baerii* and zebrafish *Danio rerio*,  
136 was this technique applied to explore the impact of diet on the  
137 gut bacterial community (Desai et al., 2012; Semova et al.,  
138 2012; Geraylou et al., 2013). In sea bream, *Sparus aurata*, data  
139 on gut bacterial community using NGS have been recently  
140 published regarding fish fed exclusively fishmeal or vegetable  
141 protein based diets (Estruch et al., 2015), while no data are  
142 available for this species fed increasing SBM levels in practical  
143 diet formulations.

144 Furthermore few studies have explored in this species the  
145 effects of increasing levels of SBM on performance using low  
146 FM based diets as the only animal protein source and most of  
147 the data on literature were restricted to replace FM with SBM.  
148 At this regards, we evaluated the effects of SBM by replacing a  
149 mixture of vegetal ingredients, wheat meal (WM), wheat gluten  
150 (WG), corn gluten (CG) and sunflower meal (SM) which are



151 currently used in practical formulation at industrial level to  
152 determine the optimal inclusion rate in practical low fish meal  
153 diet.

154 The aims of this study were: 1) to evaluate the effects of  
155 dietary inclusion of SBM and a low FM content in practical  
156 diet formulations on growth, nutrient utilization and gut  
157 histology of gilthead sea bream; 2) to evaluate changes in the  
158 gut bacterial community of gilthead sea bream fed practical  
159 diets with increasing levels of SBM and a low FM content, in  
160 comparison to a control diet.

161

## 162 **2. Materials and methods**

163

### 164 *2.1. Diets*

165

166 Ingredients and proximate composition of the experimental  
167 diets are presented in Table 1. Four isoproteic and isolipidic  
168 diets were formulated with practical ingredients to contain  
169 increasing levels of SBM (0, 100, 200, and 300 g kg<sup>-1</sup>, named  
170 S0, S10, S20, and S30, respectively) with a low FM content  
171 (150 g kg<sup>-1</sup>), while a control diet (C) was formulated to contain  
172 0 g kg<sup>-1</sup> SBM and 350 g kg<sup>-1</sup> FM content. SBM was replaced  
173 by adding WM, WG, CG and SM. The diets were  
174 manufactured by Skretting Aquaculture Research Centre  
175 (Stavanger, Norway) using extrusion technology. According to

176 the feed manufacturer, the protein and lipid levels were within  
177 the range of the commercial diets for sea bream as well as the  
178 FM level in the C group which was chosen as optimal standard  
179 level for commercial diet of this species. All feeds were  
180 produced as extruded sinking pellets (specific gravity 1.15)  
181 with a diameter of 4 mm.

182

## 183 *2.2. Fish, experimental set-up and sampling*

184

185 The experiment was carried out at the Laboratory of  
186 Aquaculture, Department of Veterinary Medical Sciences of the  
187 University of Bologna, Cesenatico, Italy. Sea bream with an  
188 initial average weight of  $75.9 \pm 1.9$  g were obtained from the  
189 hatchery Panittica Italia, Fasano, Italy. Before the experiment,  
190 fish were acclimated for 2 weeks to the experimental tanks and  
191 fed a mix of the experimental diets. At the beginning of the  
192 trial, 60 fish per tank were randomly distributed into 15, 1000 L  
193 square conical bottom tanks to obtain five triplicate fish groups,  
194 each per dietary treatment. Tanks were provided with natural  
195 seawater and connected to a closed recirculation system  
196 consisting of a mechanical sand filter (Astralpool, Spain), an  
197 ultraviolet light (Philips, the Netherlands) and a biofilter  
198 (Astralpool, Spain). The water exchange rate within each tank  
199 was 100% every hour. The water renewal of the total system  
200 was 5 % daily. Mean water temperature was maintained at 23.0

201  $\pm 1.0$  °C throughout the experiment; photoperiod was held  
202 constant at a 12 h day length through artificial light (300 lux at  
203 the water surface — Delta Ohm luxmeter HD-9221; Delta-  
204 Ohm, Padua, Italy). The oxygen level was kept constant ( $8.0 \pm$   
205  $1.0$  mg L<sup>-1</sup>) by a liquid oxygen system connected to a software  
206 controller (B&G Sinergia snc, Chioggia, Italy). Ammonia (total  
207 ammonia nitrogen, TAN  $\leq 0.1$  mg L<sup>-1</sup>), nitrite (NO<sub>2</sub>  $\leq 0.2$  mg  
208 L<sup>-1</sup>) and nitrate (NO<sub>3</sub>  $\leq 50$  mg L<sup>-1</sup>) were determined  
209 spectrophotometrically once a day (Spectroquant Nova 60,  
210 Merk, Lab business) at 12.00 p.m. At the same time, pH (7.8–  
211 8.2) and salinity (28-33 g L<sup>-1</sup>) were determined. The feeding  
212 trial lasted a total of 100 days. Fish were overfed by automatic  
213 feeders twice a day with a 5-10 % overfeeding ration for six  
214 days a week, while one meal was supplied on Sundays. Each  
215 meal lasted 1 hour and after that the uneaten feed was trapped  
216 by a feed collector at the water output of tanks, dried overnight  
217 at 105°C and the weight deducted from the feed intake for  
218 overall calculations.

219 At the beginning and at the end of the experiment, all the  
220 fish of each tank were individually weighed. At the end of the  
221 trial digesta samples from 3 fish per tank were collected  
222 individually. The gastrointestinal tract was dissected under  
223 sterile conditions and the distal gut content was squeezed out  
224 into an Eppendorf tube (one per fish) and placed at -80 °C until  
225 DNA extraction (Desai et al., 2012).

226 Carcass proximate composition was determined on a pooled  
227 sample of ten fish collected at the beginning of the trial and on  
228 pooled samples of five fish per tank collected at the end of the  
229 trial. Furthermore, at the end of the trial, wet weight of viscera  
230 and liver was individually recorded from five fish per tank to  
231 determine visceral (VSI) and hepatosomatic (HSI) indices.

232 All experimental procedures were evaluated and approved  
233 by the Ethical-scientific Committee for Animal  
234 Experimentation of the University of Bologna, in accordance  
235 with the European directive 2010/63/UE on the protection of  
236 animals used for scientific purposes.

237

### 238 2.3. *Gut histology*

239

240 At the end of the trial 15 animals per treatment were  
241 randomly sampled. After euthanasia with a lethal dose of 2-  
242 phenoxyethanol, the gut was removed and the intestine was  
243 divided into two segments, proximal and distal; from each  
244 segment a 5 mm-long piece was sectioned and fixed in 10%  
245 buffered formalin. Samples were then processed for routine  
246 histology to obtain 3 µm thick transverse sections, which were  
247 stained with haematoxylin-eosin (H&E). Sections were  
248 evaluated under a light microscope (Nikon Eclipse 80i).

249

### 250 2.4. *Gut bacterial community 16S sequencing*

251  
252       Total bacterial DNA was extracted from a pool of distal  
253 intestine content obtained from 3 fish per tank (100 mg of distal  
254 intestine content per fish) as reported by Schnorr et al. (2014).  
255 PCR amplifications of the V3-V4 region of the 16S rRNA gene  
256 were carried out in 25 µl volumes with 25 ng of microbial  
257 DNA, 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems),  
258 and 200 nM of the primers S-D-Bact-0341-b-S-17/S-D-Bact-  
259 0785-a-A-21 (Klindworth et al., 2013) including Illumina  
260 overhang adapters. Reaction conditions were as follows: initial  
261 denaturation at 98°C for 3 min, followed by 30 cycles of  
262 denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec,  
263 and extension at 72°C for 30 sec, with a final extension step at  
264 72°C for 5 min. Amplicons were purified using Agencourt  
265 AMPure XP magnetic beads. This magnetic bead-based system  
266 is recommended in the Illumina protocol “16S Metagenomic  
267 Sequencing Library Preparation” for the MiSeq system, and has  
268 been used in several other publications (Soverini et al., 2016).  
269 According to the Illumina protocol, 20% PhiX control was  
270 used. Indexed libraries were prepared by using Nextera  
271 technology and cleaned up with Agencourt® magnetic beads.  
272 The final libraries were pooled at equimolar concentrations,  
273 denatured and diluted to 6 pM before loading onto the MiSeq  
274 flow cell. Sequencing was performed on Illumina MiSeq  
275 platform using a 2 × 300 bp paired end protocol, according to

276 the manufacturer's instructions (Illumina, San Diego, CA).  
277 Raw sequences were processed using the QIIME pipeline  
278 (Caporaso et al., 2010). After length (minimum/maximum =  
279 300/600 bp) and quality filtering with default parameters, reads  
280 were binned into OTUs at a 0.97 similarity threshold using  
281 UCLUST (Edgar, 2010). Assignment was carried out by using  
282 the RDP classifier against Greengenes database (May 2013  
283 version). Alpha-diversity rarefaction curves were performed  
284 using the Faith's phylogenetic diversity, Chao1, observed  
285 species, and Shannon index metrics. Beta-diversity was  
286 estimated by weighted and unweighted UniFrac distances,  
287 which were used as input for principal coordinates analysis  
288 (PCoA).

289

## 290 *2.5. Analytical methods*

291

292 Diets and whole body samples were analyzed for proximate  
293 composition. Moisture content was obtained by weight loss  
294 after drying samples in a stove at 105 °C until a constant  
295 weight was achieved. Crude protein was determined as total  
296 nitrogen (N) by using the Kjeldahl method and multiplying N  
297 by 6.25. Total lipids were determined according to Bligh and  
298 Dyer's (1959) extraction method. Ash content was estimated by  
299 incineration to a constant weight in a muffle oven at 450 °C.

300

301    *2.6. Calculations*

302

303    The formulae employed were as follows:

304        Specific growth rate (SGR) ( $\text{day}^{-1}$ ) =  $100 * (\ln \text{FBW} - \ln$   
305    IBW)/days (where FBW and IBW represent the final and the  
306    initial body weights).

307        Feed intake (FI) ( $\% \text{ day}^{-1}$ ) =  $100 * (\text{crude feed intake}/$   
308    ABW/day) (where ABW (g) = average body weight = (FBW +  
309    IBW)/2).

310        Feed conversion ratio (FCR) = feed intake/weight gain.

311        Visceral somatic index (VSI) (%) =  $100 * (\text{viscera}$   
312    weight/body weight).

313        Hepatosomatic index (HSI) (%) =  $100 * (\text{liver weight/body}$   
314    weight).

315        Protein efficiency ratio (PER) =  $(\text{FBW} - \text{IBW})/\text{protein}$   
316    intake.

317        Gross protein efficiency (GPE) (%) =  $100 * [(\% \text{ final body}$   
318    protein \* FBW) - (% initial body protein \* IBW)]/total protein  
319    intake  $\text{fish}^{-1}$ .

320        Gross lipid efficiency (GLE) (%) =  $100 * [(\% \text{ final body}$   
321    lipid \* FBW) - (% initial body lipid \* IBW)]/total lipid intake  
322     $\text{fish}^{-1}$ .

323

324    *2.7. Statistics*

325

326 Data of growth performance, VSI, HSI, and nutritional  
327 indices are presented as mean  $\pm$  standard deviation (SD) of  
328 three replicate groups and were analyzed by a one-way  
329 ANOVA followed by a Tukey's multiple comparison test.  
330 Statistical analysis of gut bacterial community was carried out  
331 by using R packages Stats and Vegan. Significant differences in  
332 the relative abundance of gut bacterial community components  
333 were obtained by Kruskal-wallis test. Data separation in the  
334 PCoA was tested using a permutation test with pseudo F-ratios  
335 (function Adonis in the Vegan package).

336

### 337 **3. Results**

338

#### 339 *3.1. Growth and histology*

340

341 Growth performance is summarized in Table 2. At the end  
342 of the trial fish fed the S30 diet showed a higher ( $P \leq 0.05$ )  
343 SGR compared to S0 and a higher FI compared to S0, S10 and  
344 S20. Sea bream fed the C diet had a higher ( $P \leq 0.05$ ) FI  
345 compared to S0, while no significant differences in FCR among  
346 the treatments were detected (Table 2). No significant  
347 differences in VSI, HSI, whole body composition and the  
348 nutritional indices PER, GPE, GLE, were observed among the  
349 treatments (Table 3). No specific histopathological changes



indicative of soy-induced enteritis were observed in the intestine of any fish examined (Fig. 1).

### *3.2. Gut bacterial community characterization*

Fifteen pools of distal intestine content were analyzed by NGS of the V3 and V4 regions of the 16S rDNA gene. A total of 5,584,914 high quality reads were obtained from the starting 15,956,896 reads obtained, ranging from a minimum of 93,673 to a maximum of 687,596 reads per sample, with an average of 372,327 reads per sample. Further information about the number of reads for each sample and the coverage are reported in Supplementary Table 1. The number of reads across samples was normalized basing on the sample with the lowest number of reads and singletons were omitted from the analysis. Reads were clustered into 13,099 operational taxonomic units (OTUs) at 97% of identity, of which a total of 5,525 diet-specific OTUs were found (1,082 for diet S30; 1,016 for diet S20; 1,038 for diet S10; 833 for diet S0; 1,556 for control diet). Different metrics have been utilized to calculate  $\alpha$ -diversity, including phylogenetic diversity, OTU species count, Chao 1 index for microbial richness and Shannon index for biodiversity (Fig. 2a). Rarefaction curves of the phylogenetic diversity approximated saturation, indicating a good coverage of the gut bacterial community. No differences in the gut bacterial

community  $\alpha$ -diversity according to the different diets were detected (Fig. 2b).

At the phylum level, the average sea bream gut bacterial community is dominated by *Firmicutes* (relative abundance (rel. ab.) 71%), *Actinobacteria* (rel. ab. 9%), *Bacteroidetes* (rel. ab. 7%) and *Proteobacteria* (rel. ab. 6%), while *Cyanobacteria* (rel. ab. 3%) and *Verrucomicrobia* (rel. ab. 3%) were subdominant (Fig. 3a). The most represented families are: *Lactobacillaceae* (rel. ab. 26%), *Ruminococcaceae* (rel. ab. 12%), *Lachnospiraceae* (rel. ab. 10%) and *Clostridiales* families (rel. ab. 7%) (Fig. 3b). Among the subdominant families the most represented were, *Streptococcaceae* (rel. ab. 3%), *Cyanobacteria* (rel. ab. 3%), *Staphylococcaceae* (rel. ab. 3%), *Verrucomicrobia* (rel. ab. 3%) and *Enterobacteriaceae* (rel. ab. 2 %).

In order to highlight the impact of the different diets (S0, S10, S20, S30 and C) on the gut bacterial ecology of sea bream, we performed the PCoA analysis of the UniFrac distances among the gut bacterial community profiles (Fig. 4). Even though no significant differences among dietary groups were detected, both weighted and unweighted PCoA showed a tendency toward a samples separation according to the different diets. Fig. 5 shows the relative abundance of bacteria composition per sample at phylum (a) and family (b) levels, while in Fig. 6 we report the gut bacterial community components which showed

a different abundance in the different dietary groups. In particular, the abundance of *Cyanobacteria* progressively increased from diet C to diet S30 (Fig. 6a), while *Synergistetes* tend to show an opposite trend (Fig. 6b). Differently, *Actinobacteria* showed a higher abundance in diets S0 and S30 (Fig. 6c). Although there were no statistically significant effects, the *Lactobacillaceae* family was highly represented in fish fed S30 (Rel. ab. 43.3%) compared to those fed C diet (Rel. ab. 11.2%) (Fig. 6d).

#### 4. Discussion

The inclusion of SBM at 100, 200 and 300 g kg<sup>-1</sup> (S10-S30) of the diet with a low FM content (150 g kg<sup>-1</sup>) led to equal growth and protein utilization in comparison to a control diet without SBM and having 350 g kg<sup>-1</sup> of FM. The present results are in agreement with previous studies which have demonstrated the feasibility of including up to 300, 390 and 395 g kg<sup>-1</sup> SBM in diets for on-growing sea bream without negative effects on growth and nutritive efficiency (Bonaldo et al., 2008; Martinez-Llorens et al., 2009; Kokou et al., 2012), although FM levels in these studies were higher than in the present trial or amino acid supplements were used. In the present study the lack of differences in the SGR, FCR, PER and GPE between S10, S20, S30, the C diet suggests that the

inclusion of 150 g kg<sup>-1</sup> of FM in combination with SBM, WG and CG will supply sufficient protein quality for this species. Similarly, Dias. et al. (2009), showed that the growth performance of sea bream towards the end of the grow-out phase can be sustained by a practical dietary formulation containing plant protein-derived and as little as 13% of marine-derived proteins. However, in that study AA supplementation and haemoglobin powder were also incorporated in the feed while in the present study FM was the only animal protein source. Focusing on the diets at low FM level (S0, S10, S20, S30), fish fed S30 showed a higher SGR compared to those fed S0. This seems mainly due to an increment of FI with increasing dietary content of SBM. The reduced FI commonly observed in fish given feeds containing plant protein may be related to a reduced feed palatability and, in this regard, the use of several mixtures of plant protein should reduce the potential inhibition of feed consumption due to the specific effect of a single ingredient (Fournier et al., 2004). Other studies reported an increased feed consumption with increasing dietary levels of SBM assuming that fish to meet their energy needs would have increased the FI for a reduced available energy content as SBM inclusion increased (Venou et al., 2006; Kokou et al., 2012). SBM contains about 20% of non-starch polysaccharides (NSP) and 10% oligosaccharides (Snyder and Kwon, 1987; Bach Knudsen, 1997), which are considered indigestible by fish

450 compared to wheat and glutsens. Therefore, despite the  
451 isoenergetic content of the diets, a reduction of available energy  
452 content would be expected at higher SBM inclusion level  
453 (Kokou et al., 2012). However, possible action of the gut  
454 bacterial community could allow part of SBM energy  
455 originating from NSP to be available to the fish in the form of  
456 low molecular weight fatty acids (Kihara and Sakata, 2002;  
457 Mountfort et al., 2002; Refstie et al., 2005; Kokou et al., 2012).

458 Gut histology revealed no specific histopathological changes  
459 indicative of soy-induced enteritis in the intestines of any fish  
460 examined. In a previous study on sea bream the inclusion of  
461 30% SBM seemed to cause moderate and diffused expansion of  
462 lamina propria in the distal intestine due to an increase of  
463 mononuclear cell infiltration when compared to other  
464 treatments with 18 and 0% of SBM (Bonaldo et al., 2008). A  
465 dilatation of the submucosa by eosinophilic cells infiltration  
466 was also found in the distal intestine of sea bream fed diet  
467 containing bioprocessed SBM at the 40 and 60% levels (Kokou  
468 et al., 2012). However both studies were conducted at juveniles  
469 stage (weight range, 17.4 - 96.0 g and 15.7 - 48.9 g,  
470 respectively) compared to the on-growing stage of the present  
471 study (weight range 75.1 - 259.5 g). The inclusion levels of  
472 SBM seem to be better tolerated by fish at on-growing phase as  
473 supported by Martinez-Llorens et al. (2007) which concluded  
474 that dietary SBM might be included in the diets up to 30% in

475 juveniles and up to 50% in grow-out fish without affecting  
476 animal performance. In addition sea bream in grow-out phase  
477 showed high tolerance for soy saponins while in juvenile sea  
478 bream fed diets containing phytosterols and soy saponins some  
479 disturbances of the intestinal mucosa were observed (Couto et  
480 al., 2014 a, b); however, the histomorphological changes  
481 observed were very mild and, although statistically significant,  
482 the differences were judged to be minor and to represent  
483 normal adaptation to changes in diet composition (Couto et al.,  
484 2014a).

485 In the present study the gut bacterial community was  
486 characterized. According to our findings, the gut bacterial  
487 community is widely dominated by *Firmicutes* (rel. ab. 71%),  
488 showing *Actinobacteria* as the second dominant phyla (rel. ab.  
489 9%). *Bacteroidetes*, *Proteobacteria* and *Cyanobacteria* were  
490 subdominant components with a relative abundance ranging  
491 from 3 to 7 % of the bacterial community. Our data are in  
492 general agreement with the previous Next Generation  
493 Sequencing-based survey of the gut bacterial community in sea  
494 bream (Estruch et al., 2015). Further, by mean of  
495 pyrosequencing of the V1-V3 region of the 16S rDNA, the  
496 Authors showed a co-dominance of *Actinobacteria* (rel. ab.  
497 35%), *Proteobacteria* (rel. ab. 32%) and *Firmicutes* (rel. ab.  
498 24%) in the hindgut bacterial community. The dominance of  
499 *Firmicutes* we observed in the sea bream analyzed in the

500 present study may be imputed to their specific dietary regimen  
501 and rearing conditions, which represent environmental  
502 variables known to mold the compositional structure of the gut  
503 bacterial community. According to our findings, the gut  
504 bacterial community of sea bream was enriched in several  
505 fibrolytic *Firmicutes*, such as *Ruminococcaceae*,  
506 *Lachnospiraceae* and *Clostridiales*. By producing butyrate  
507 from indigestible complex polysaccharides, these  
508 microorganisms may provide important beneficial functions for  
509 the host (Nicholson et al., 2012). Indeed, butyrate plays  
510 multiple roles in host physiology, being strategic for the  
511 amelioration of energy extraction from diet, for the  
512 reinforcement of the gut epithelium barrier as well as for  
513 modulation of the host immune function (Petersson et al., 2011;  
514 Arpaia et al., 2013; Russell et al., 2013). In addition, our  
515 finding of *Cyanobacteria* in the sea bream gut bacterial  
516 community is of particular interest in the context of the recent  
517 findings by Di Rienzi et al. (2013). The Authors performed the  
518 first whole genome reconstruction of *Cyanobacteria* detected in  
519 the gut and proposed their specific designation as a new  
520 candidate sibling phylum named *Melainabacteria*. Differently  
521 from environmental *Cyanobacteria*, gut *Melainabacteria* are  
522 non-photosynthetic and non-respiratory, while, according to the  
523 authors, these microorganisms are obligate anaerobic  
524 fermenters capable to relay on the different carbon sources

525 present in the gut. Analogous to certain *Firmicutes*,  
526 *Melainabacteria* can ferment plant polysaccharides in the gut,  
527 and being able to provide the host with B and K vitamins, these  
528 microorganisms have been included among the mutualistic  
529 components of the gut bacterial community (Di Rienzi et al.,  
530 2013).

531 Our finding showed only a subtle impact for the different  
532 diets on the overall gut bacterial composition of sea bream, as  
533 shown by PCoA analysis. However, evidence suggesting the  
534 impact of different levels of SBM on specific components of  
535 the gut bacterial community was obtained. At phylum level,  
536 increasing SBM dietary levels seem to favor the increase of  
537 *Cyanobacteria* and a correspondent decrease in *Synergistetes*.  
538 While the first is considered as a mutualistic gut bacterial  
539 community component able to provide the host with essential  
540 vitamins, *Synergistetes* act as opportunistic pathogens in the gut  
541 (Marchandin et al., 2010). Moreover, within the phylum of  
542 *Firmicutes* the fish fed a high level of SBM (S30) were  
543 enriched with the family of *Lactobacillaceae*, compared to  
544 those fed the control diet. The functional impact of lactic acid  
545 bacteria on fish intestine is still unclear, but potentially they  
546 may have beneficial effects on the immune system, could  
547 protect the fish against pathogenic invasion through the  
548 intestinal surface, are probiotic candidates and are generally  
549 considered as organisms associated with a healthy intestinal



550 epithelium (Cai et al., 1998; Nayak, 2010; Salinas et al., 2008;  
551 Dimitroglou et al., 2009; Ingerslev et al., 2014). Interestingly,  
552 in rainbow trout, Wong et al. (2013) described a trend of taxa  
553 within the phylum *Firmicutes* that were significantly  
554 discriminatory for diet type in which the relative abundance of  
555 *Lactobacillaceae* was enriched in fish fed a grain-based diet.  
556 Also the cichlid, *Astatotilapia burtoni*, which mostly feeds on  
557 plants and algae, exhibited most of the gut microbial  
558 biodiversity seen in cichlids with several nearly exclusive  
559 bacterial taxa such as *Lactobacillales* and gut *Melainabacteria*  
560 (Baldo et al., 2015).

561 What favors the presence of *Lactobacillaceae* in fish fed a plant  
562 diet is not well known, but some studies have shown that  
563 polyunsaturated fatty acids depress the intestinal lactobacilli  
564 population in fish (Ringø, 1993) in accordance with the more  
565 recent finding of Ingerslev et al. (2014), where a significantly  
566 lower amount of lactic acid bacteria was found in rainbow trout  
567 fed a marine-based diet compared to the fish fed a plant-based  
568 diet containing rape seed oil and pea meal. In contrast, in sea  
569 bream total fishmeal replacement with plant protein had a  
570 negative effect on the relative abundance of *Firmicutes*  
571 throughout the gut, particularly on the lactic acid bacteria  
572 *Lactobacillus* and *Streptococcus* (Estruch et al., 2015).  
573 *Lactobacillus* species are well equipped to metabolize  
574 oligosaccharides that occur in their habitats, such as sucrose,

575 stachyose and raffinose which are contained in soybeans at  
576 approximately 10 % (Espinosa-Martosy and Rupérez, 2006;  
577 NRC, 2011; Gänzle and Follador, 2012). Moreover,  
578 *Lactobacillus* can benefit from simple sugars derived from  
579 primary degraders in the gut, establishing syntrophic networks.  
580 Thus, in the context of our research, it is reasonable to  
581 hypothesize that the *Lactobacillaceae* growth could be  
582 supported by these oligosaccharides.

583

## 584 **5. Conclusion**

585

586 In conclusion results of growth, nutrient utilization and gut  
587 histology indicate that SBM can be successfully incorporated  
588 up to a level of 300 g kg<sup>-1</sup> with the inclusion of 150 g kg<sup>-1</sup> of  
589 FM as the only animal protein source, without any deleterious  
590 effects on growth, protein utilization and gut health during the  
591 on-growing phase.

592 A deep sequencing of the gut bacterial community of sea  
593 bream during the on-growing phase was successfully obtained.  
594 For the first time in this species, the gut bacterial community  
595 was analyzed by NGS in fish fed increasing SBM levels using  
596 practical current formulations. The overall gut bacterial  
597 community was largely dominated by *Firmicutes*, including  
598 several fibrolytic bacteria, supporting the hypothesis that this  
599 species could be predisposed to digest plant-based ingredients.

600 A minimal impact of increasing dietary SBM levels on the  
601 overall gut bacterial community was observed. However SBM  
602 seems to favor positively specific components of the gut  
603 bacterial community such as *Cyanobacteria* and  
604 *Lactobacillaceae* which may provide important beneficial  
605 functions for the host and be associated with a healthy intestinal  
606 epithelium.

607

#### 608 **Conflicts of interest**

609

610 The authors declare no conflicts of interest.

611

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613

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#### 617 **References**

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 862

863 **Figure captions**

864 Figure 1: histology of sea bream foregut (a,c,e,g,i) and hindgut  
865 (b,d,f,h,l). Control diet, C (a,b); 0 g kg<sup>-1</sup> SBM diet, S0 (c,d);  
866 100 g kg<sup>-1</sup> SBM diet, S10 (e,f); 200 g kg<sup>-1</sup> SBM diet, S20 (g,h)  
867 and 300 g kg<sup>-1</sup> SBM diet, S30 (I,l). Intestine does not show any  
868 differences in terms of inflammatory or degenerative changes  
869 among diets (H&E, 20x objective).

870 Figure 2 a, b: OTUs rarefaction curves carried out with  
871 different  $\alpha$ -diversity metrics (Faith's phylogenetic diversity  
872 (PD whole tree), observed OTUs, the Chao1 measure of  
873 microbial richness, and the Shannon index of biodiversity).

874 Figure 3: sea bream gut bacterial community composition at  
875 phylum (a) and family levels (b).

876 Figure 4: weighted and unweighted UniFrac distance PCoA of  
877 the gut bacterial community of sea bream treated with different  
878 diets, color code: S30 diet red, S20 diet green, S10 diet yellow,  
879 S0 diet blue, C diet purple. MDS1 and MDS1 represent the  
880 15.4 and 2.6 % of the total variability, respectively.  
881 Permutation test with pseudo F-ratios:  $P = 0.107$  and  $P = 0.091$   
882 for weighted and unweighted UniFrac, respectively.

883 Figure 5: relative abundance of bacteria composition per  
884 sample at phylum (a) and family levels (b).

885 Figure 6: box plot showing the relative abundance of (a)  
886 *Cyanobacteria*, (b) *Synergistetes*, (c) *Actinobacteria* and (d)

887 *Lactobacillaceae* in different diets. Significance of the  
888 differences was obtained by Kruskal-Wallis test.

889

890

**Table 1.** Formulation and proximate composition of the experimental diets

<i>Ingredients (g kg<sup>-1</sup>)</i>	S0	S10	S20	S30	C
FM North Atlantic	150	150	150	150	350
Hi Pro SBM	0	100	200	300	0
Wheat meal	206.4	165.6	125.8	84.0	229.3
Wheat gluten	226	199.1	175.9	150	127.7
Corn gluten	200	185	165	150	130
Sunflower meal	80	60	40	20	40
Fish oil North Atlantic	132.5	135.3	138.3	141	118
Vit/Min premix*	5	5	5	5	5
<i>Proximate composition (g kg<sup>-1</sup>)</i>					
Moisture	77	76	78	80	60
Crude protein	466	466	479	478	460
Crude fat	194	192	199	209	197
Ash	45	47	48	57	69

FM, fishmeal; SBM, soybean meal; S0, 0 g kg<sup>-1</sup> SBM diet; S10, 100 g kg<sup>-1</sup> SBM diet; S20, 200 g kg<sup>-1</sup> SBM diet; S30, 300 g kg<sup>-1</sup> SBM diet; C, control diet.

\*Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011).

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**Table 2.** Growth performance of sea bream fed the experimental diets

	Experimental diet				
	S0	S10	S20	S30	C
<i>Growth</i>					
IBW (g)	76.0 ± 1.6	75.1 ± 0.6	77.1 ± 3.1	76.7 ± 1.9	74.4 ± 0.9
FBW (g)	249.1 ± 6.1	249.2 ± 3.9	257.6 ± 6.2	259.5 ± 5.9	256.2 ± 5.8
SGR (day <sup>-1</sup> )	1.17 ± 0.03 <sup>a</sup>	1.20 ± 0.01 <sup>ab</sup>	1.22 ± 0.01 <sup>ab</sup>	1.25 ± 0.01 <sup>b</sup>	1.21 ± 0.04 <sup>ab</sup>
FI (% day <sup>-1</sup> )	1.40 ± 0.01 <sup>a</sup>	1.45 ± 0.01 <sup>ab</sup>	1.44 ± 0.03 <sup>ab</sup>	1.51 ± 0.03 <sup>c</sup>	1.46 ± 0.02 <sup>bc</sup>
FCR	1.33 ± 0.03	1.35 ± 0.01	1.33 ± 0.01	1.36 ± 0.04	1.36 ± 0.05

S0, 0 g kg<sup>-1</sup> soybean meal SBM diet; S10, 100 g kg<sup>-1</sup> SBM diet; S20, 200 g kg<sup>-1</sup> SBM diet; S30, 300 g kg<sup>-1</sup> SBM diet; C, control diet. IBW, initial body weight; FBW, final body weight; SGR, specific growth rate, 100 \* (ln FBW - ln IBW) / days; FI, feed intake, 100 \* (crude feed intake / ((FBW + IBW) / 2) / days; FCR, feed conversion rate, (feed intake / weight gain).

Data are given as the mean (n=3; n=60 for IBW and FBW) ± SD. In each line, different superscript letters indicate significant differences among treatments ( $P \leq 0.05$ ).

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**Table 3.** Viscerosomatic index, hepatosomatic index, body composition and nutritional indices of sea bream fed the experimental diets.

<i>Experimental diet</i>					
	S0	S10	S20	S30	C
VSI	5.62 ± 0.83	5.98 ± 0.95	5.99 ± 0.93	5.72 ± 0.70	5.78 ± 1.03
HSI	1.70 ± 0.34	1.60 ± 0.31	1.64 ± 0.33	1.59 ± 0.32	1.59 ± 0.35
<i>Whole body composition (g kg<sup>-1</sup>)</i>					
Moisture	619 ± 4.7	626 ± 6.2	628 ± 4.6	632 ± 1.3	615 ± 5.1
Crude protein	174 ± 2.4	174 ± 2.6	175 ± 2.6	179 ± 0.9	173 ± 0.7
Total lipids	173 ± 9.3	175 ± 7.1	175 ± 9.2	180 ± 5.6	174 ± 6.0
Ash	33 ± 2.1	33 ± 1.3	32 ± 2.5	30 ± 0.7	33 ± 2.0
<i>Nutritional indices</i>					
PER	1.62 ± 0.04	1.59 ± 0.01	1.58 ± 0.03	1.54 ± 0.04	1.60 ± 0.06
GPE	28.8 ± 0.92	28.2 ± 0.50	28.4 ± 1.07	28.3 ± 0.86	28.2 ± 1.21
GLE	69.6 ± 4.14	70.1 ± 3.98	69.8 ± 5.26	67.2 ± 4.10	70.3 ± 4.92

S0, 0 g kg<sup>-1</sup> soybean meal SBM diet; S10, 100 g kg<sup>-1</sup> SBM diet; S20, 200 g kg<sup>-1</sup> SBM diet; S30, 300 g kg<sup>-1</sup> SBM diet; C, control diet. VSI, viscerosomatic index; HSI, hepatosomatic index; PER, protein efficiency ratio; GPE, gross protein efficiency; GLE, gross lipid efficiency.

Data are given as the mean (n=3; n=15 for VSI and HSI) ± SD. In each line, different superscript letters indicate significant differences among treatments ( $P \leq 0.05$ ). PER, ((final body weight – initial body weight) / protein intake); GPE, (100\*[(% final body protein \* final body weight) – (% initial body protein \* initial body weight)] / total protein intake fish<sup>-1</sup>); GLE, (100\*[(% final body lipid \* final body weight) – (% initial body lipid \* initial body weight)] / total lipid intake fish<sup>-1</sup>).

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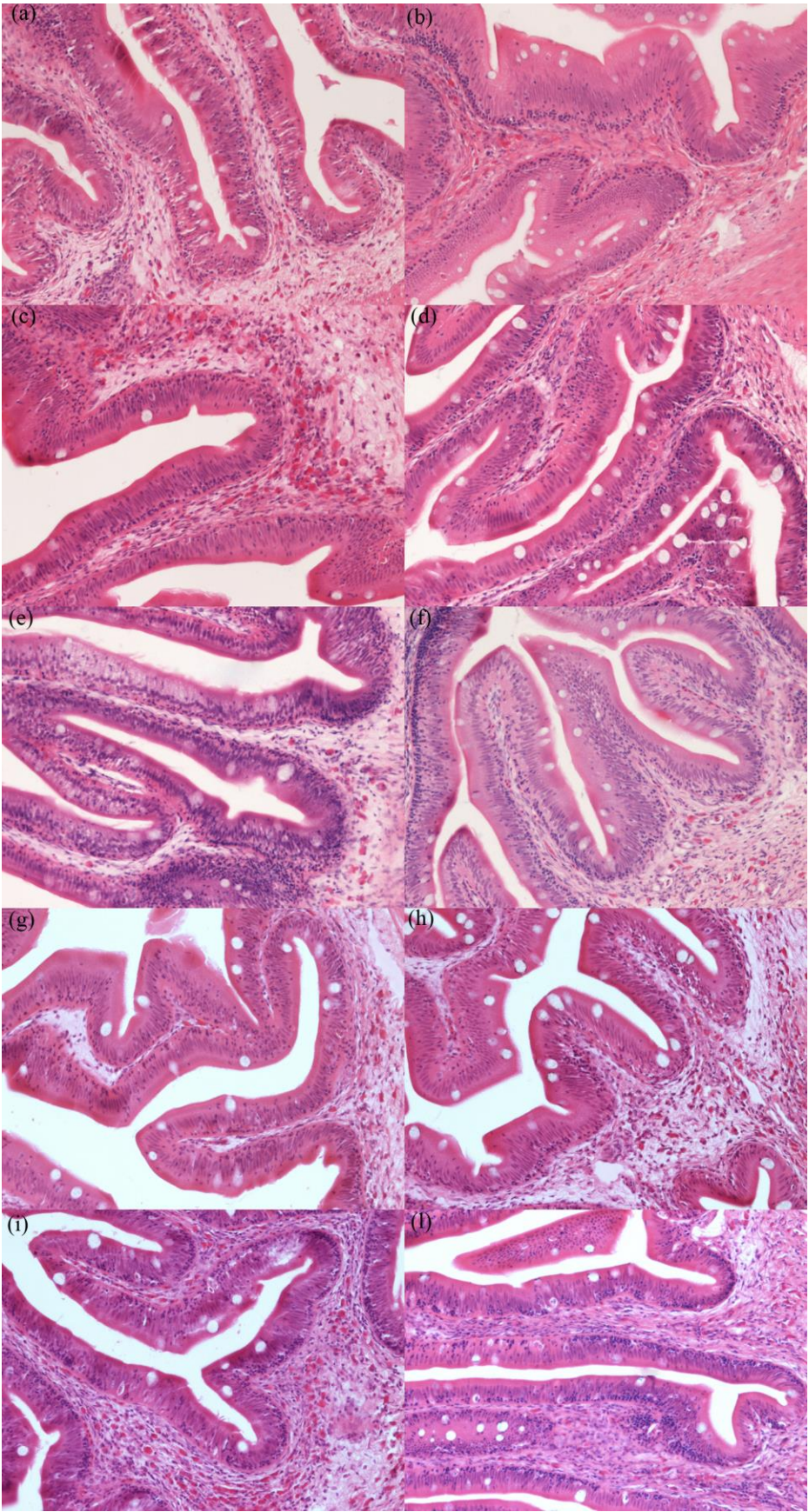
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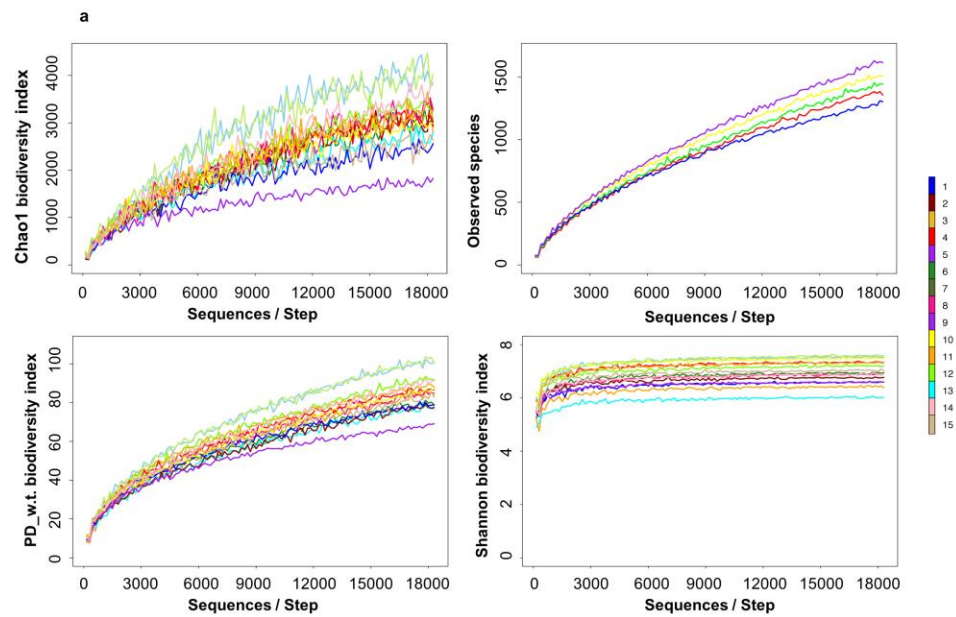
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943      Figure 2a

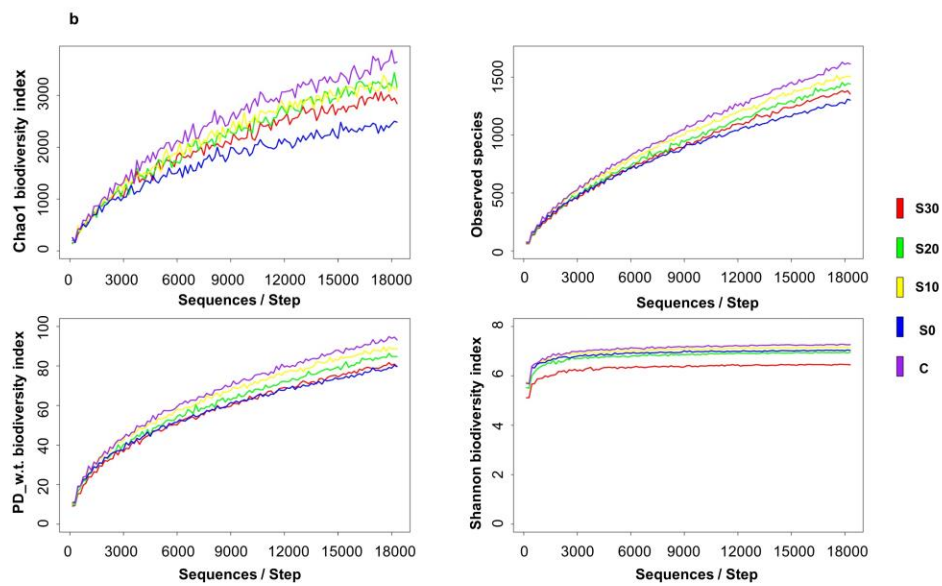


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947      Figure 2b

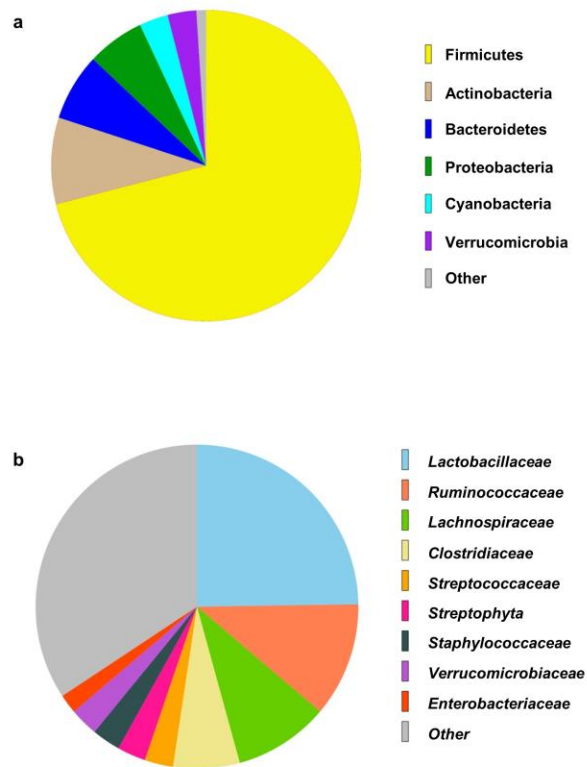


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951     Figure 3

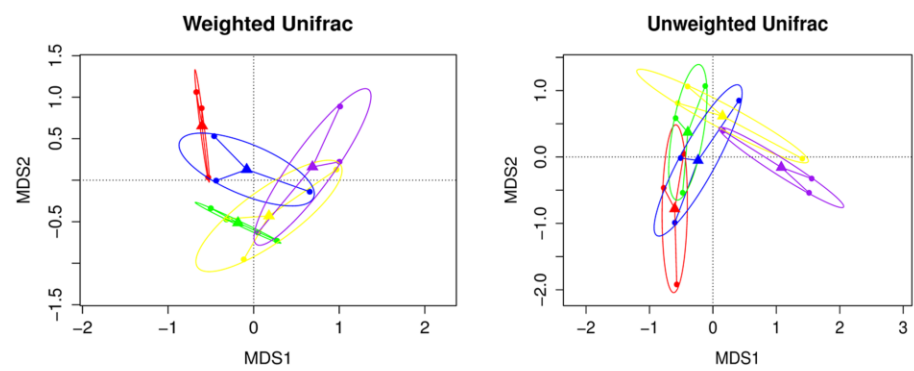


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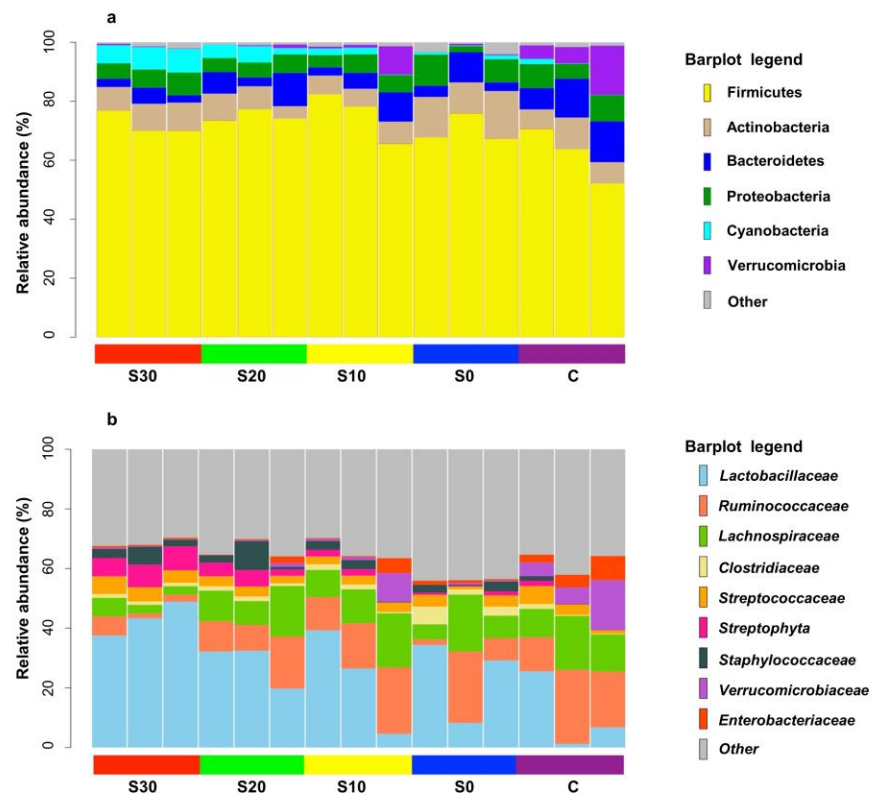
954    Figure 4



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957     Figure 5



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